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Studies on the interaction of bacterial capsular polysaccharide-*Klebsiella* K16 with cationic dyes

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Abstract

The spectral studies of cationic dyes, pinacyanol chloride and acridine orange with bioploymer, *Klebsiella* K16 capsular polysaccharide (PK16) in aqueous media reveal many interesting phenomena. The polysaccharide induces metachromasy in the dye, resulting a blue shift from 600 to 490 nm. The stoichiometry and also thermodynamic parameters of the polymer/dye in the metachromatic compound and the effect of different solvent–cosolvent on its stability have been studied. Quenching of fluorescence of acridine orange by PK16 has added another evidence for the existence of the staggered aggregation of dye. In addition to this, the equivalent weight, chromotropic character, conformation of the biopolymer and the site of interaction between dye and *Klebsiella* K16 have been pointed out.

Keywords: Dye–polymer interaction; Metachromatic compound; Reversal of metachromasy; Chromotropic property; Fluorescence quenching

1. Introduction

The bacterial polysaccharides make up a group of biopolymers in which the structural variation is almost unlimited. A majority of the bacterial polysaccharides show high degree of immunological specificity and are produced by one type or species of bacteria [1].

Klebsiella bacteria produce extracellular polysaccharides which surround the bacterium as an additional outer layer as capsule. The capsular material also exhibits specific antigenic properties and the serological classification of *Klebsiella* is based on immunological reactions of these K-antigens. The *Klebsiella* capsular antigens have been found to be safe in human and these antigen polysaccharides are now used as vaccines [2] which are nonpyrogenic and immunogenic. Due to the potential use of the bacterial polysaccharides in immunological and vaccine preparations, primary structural studies and conformational analysis as well as studies on various physico-chemical properties of these biopolymers are gaining more and more importance.

The interaction of small dye molecules with the biopolymers like cellulose [3], glycosaminoglycans of connective tissue [4] and heparins [5] have been studied. Formation of a metachromatic compound is characterized by the appearance of a blue shift in the visible absorption spectrum of the dye during dye–polymer interaction. Metachromasy is generally applied to the aggregations of cationic dye on anionic polymers [6] and it depends on the conformation of the polyanion as well as the dye ions in solution.

Studies on binding of cationic dye to different synthetic and natural polyanions by absorbance, fluorescence and circular dichromism experiments yielded significant contributions in the field of dye–polymer interaction [7,8] during the last few decades. In the last few years, chromotropic properties of different bacterial capsular polysaccharide with respect to induction of metachromasy in different cationic dyes have been investigated in our laboratory [9–12]. But the work on the interaction of *Klebsiella* K16 polymer with dye

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has not yet been reported in the literature. To get the clear idea between the interaction of *Klebsiella* K16 toward the dye, the spectral and thermodynamic properties of cationic dyes with PK16 have been studied. The equivalent weight, chromotropic character and conformation of the polymer as well as its site of interaction with dye have been highlighted in this paper.

2. Experimental

The *Klebsiella* K16 test strain was obtained from Dr. S. Schelecht, Max Planck Institute for Immunobiology, Freiburg, Germany. The dyes pinacyanol chloride (1-ethyl-2-[3-(1-ethyl-2(1*H*)quinolylidene)-propenyl]-quinolinium chloride) and acridine orange (3,6-bis[dimethyl amino]-acridinium chloride) were supplied by Sigma. The bacterial cells were grown in the laboratory and capsular polysaccharide (CPS) was isolated and purified by phenol–water–cetavlon method [13].

Absorption spectra were recorded on spectrophotometer (model M. Roy Spectronic 21D) using matched pair of quartz cells of 1 cm pathlength. The fluorescence spectra were recorded on Shimadzu spectrofluorophotometer (model RF-2000).

The equivalent weight of the CPS was determined by conductometric titration with 0.1 mol dm^{-3} NaOH solution in a Systronic conductometric bridge. Stoichiometry of the CPS–dye complex was determined by the isolation method of MacIntosh [14]. General experimental details were the same as described earlier [15].

3. Results and discussion

Klebsiella K16 CPS is found to contain D-glucose, D-galactose, L-fucose and D-glucouronic acid in an approximate molar ratio 1:1:1:1. The primary structure of the *Klebsiella* K16 (Fig. 1) consists of tetrasaccharide repeating units comprising a →3)-α-D-Glcp-(1→4)-β-D-Glc Ap-(1→4)-α-L-Fucp-(1-chain with a β-D-Galp)-(1→ branch at position 4 of the D-glucosyl residue [16]. It does not contain any pyruvic acid or *O*-acetyl substitution.

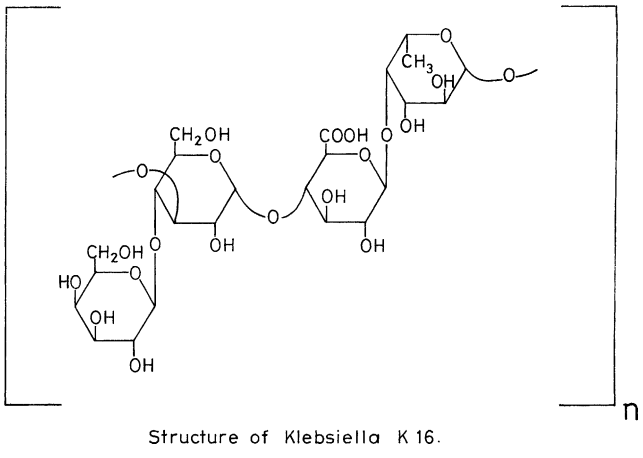

Fig. 1. The structure of *Klebsiella* K16 capsular polysaccharide.

3.1. Determination of equivalent weight of CPS

The isolated sodium salt of the capsular polysaccharide was converted into acidic form by passing the aqueous solution of the CPS through a column of Dowex 50 (H^+). The variation of conductance of acidic polysaccharide with 0.1 mol dm^{-3} NaOH solution have been computed and the equivalent weight of CPS is obtained as a point of intersection from the plot of conductance versus volume of NaOH solution. The experimentally determined value of equivalent weight is 678 ± 10 which is close to calculated value 650, based on molecular structure. The molar mass of the CPS as defined above was also determined by spectrophotometric and spectrofluorometric titration with dyes, pinacyanol chloride (PCYN) and acridine orange (AO), respectively, in the dilute solution of polymer.

3.2. Dye–polymer interaction

Aqueous solution of 1.0×10^{-5} mol dm^{-3} PCYN dye showed two sharp peaks at 600 and 550 nm corresponding to monomeric band (α) and dimeric band (β), respectively. With addition of different volume of CPS to fixed volume of PCYN dye having concentration 0.5×10^{-4} mol dm^{-3} of both, different polymer/dye (P/D) molar ratio were made and absorbances of these solutions were measured in the 450–650 nm wavelength region. The absorption spectra of dye–CPS *Klebsiella* K16 system are shown in Fig. 2. From

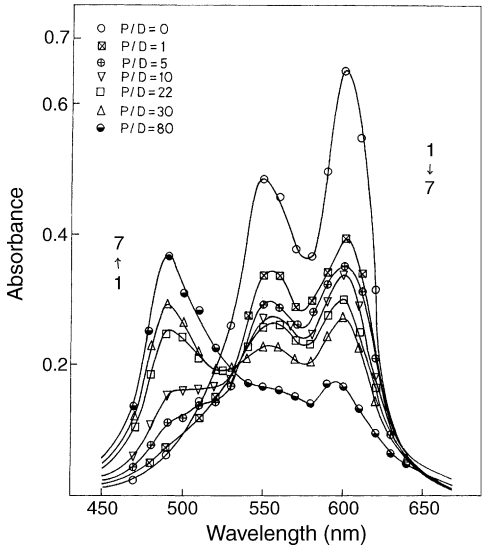

Fig. 2. The visible absorption spectra of pinacyanol chloride–*Klebsiella* K16 CPS at different CPS/dye (P/D) molar ratios at 298 K. Concentration of dye: 1.0×10^{-5} mol dm^{-3}. P/D: (1) 0.0, (2) 1.0, (3) 5.0, (4) 10.0, (5) 22.0, (6) 30.0, and (7) 80.0.

the spectra, it was found that upon addition of *Klebsiella* K16, i.e., with increase in P/D ratio, intensities of both α and β bands decreased and a new band (μ) appears at 490 nm with enhanced intensity. The blue shifted λ_{max}, i.e., μ band indicated clearly the induction of metachromasy in PCYN by the polymer. For a distinct metachromatic band, minimum P/D of 10 was necessary.

The stoichiometry of the dye–polymer complex was determined by MacIntosh method [14]. According to this method, the dye–polymer metachromatic compound was extracted in petroleum ether from the uncomplexed dye which remained in aqueous solution. The concentration of complexd dye was obtained by subtracting the free dye concentration from the initial concentration of dye, and were plotted against the concentration of the CPS. The stoichiometry of the dye and polymer in the dye–polymer complex was found to be 1:1.17 from the point of intersection of two lines.

The stoichiometry of dye–polymer complex was also determined by spectrophotometric titration in which absorbances of PCYN dye at λ_{max} 600 nm were noted with fixed concentration of dye and different concentration of CPS *Klebsiella* K16. The stoichiometry of D/P was found to be 1:0.98 and the equivalent weight 641.88 from the intersection of two lines by plotting absorbance at 600 nm versus volume of polymer solution (Fig. 3).

Based on stoichiometry of the dye–polymer system obtained from the above two methods, it can be assumed that dye and polymer forms 1:1 molecular complex. The spectrophotometric data of Fig. 2 were employed to calculate the thermodynamic properties of dye–polymer interaction. For 1:1 complex, the binding constant (K_c) can be determined by using Rose and Drago equation [17] in the following form:

$$\frac{C_D C_S}{A - A_0} = \frac{1}{K_c L(\varepsilon_{DS} - \varepsilon_D)} + \frac{C_S}{L(\varepsilon_{DS} - \varepsilon_D)} \quad (1)$$

where C_D and C_S are the initial concentrations of dye PCYN and *Klebsiella* K16 polymer, respectively; L is the optical pathlength of the solution; A and A_0 are the absorbances of the dye at the absorption maximum of the complex with and without polymer at 490 nm; and ε_{DS} and ε_D are the respective molar extinction coefficients of complex and dye at the absorption maximum of the complex. $C_D C_S/(A - A_0)$ versus C_S were plotted for PCYN and *Klebsiella* K16 in different temperature which were found to be linear, confirming 1:1 complex formation (Fig. 4). From the slope of each plot, K_c of the dye–polymer complex was calculated. The thermodynamic quantities of this complex were obtained from the equilibrium constants at different temperature by the usual method. All the thermodynamic parameters of the complex in aqueous media are presented in Table 1.

The effect of different solvent (methanol, ethanol, *n*-propanol) and other cosolvent (DMSO, DMF and urea) on the stability of CPS–dye complex have been studied by

Table 1
Thermodynamic parameters for the interaction of *Klebsiella* K16 capsular polysaccharide and pinacyanol chloride

Temperature (K)	K_c ($\times 10^{-3}$ dm^3 mol^{-1})	ΔG (kcal mol^{-1})	ΔH (kcal mol^{-1})	ΔS (cal mol^{-1} K^{-1})
303	2.40	−4.72		
308	2.06	−4.70	−5.41	−2.30
318	1.56	−4.68		
323	1.37	−4.67		

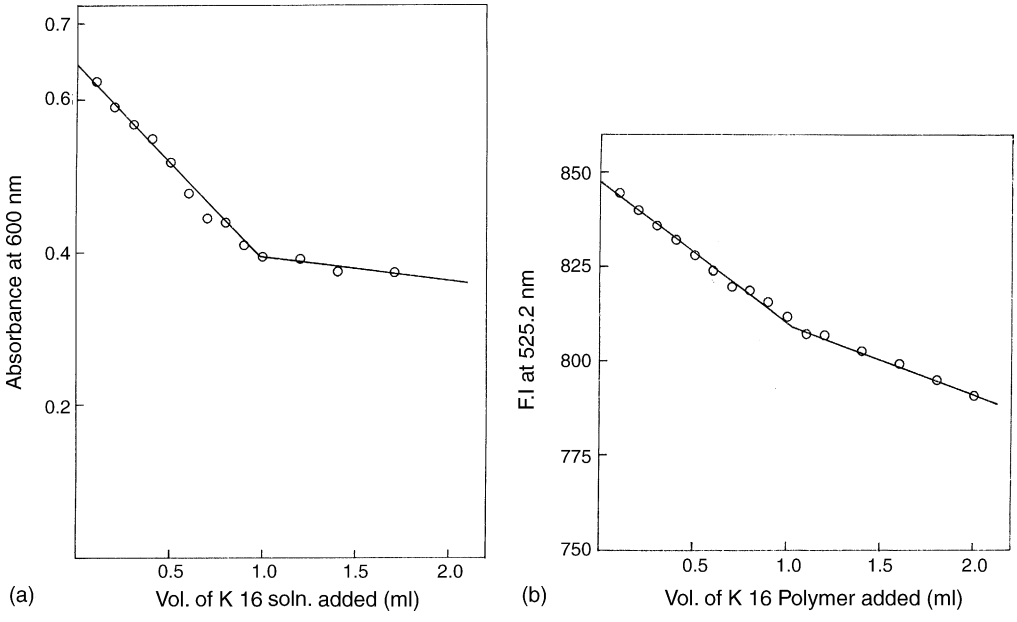

Fig. 3. (a) Spectrophotometric titration of pinacyanol chloride and (b) spectrofluorometric titration of acridine orange with *Klebsiella* K16 polymer at 298 K. Concentrations of both dye and polymer: 1.0×10^{-5} mol dm^{-3}.

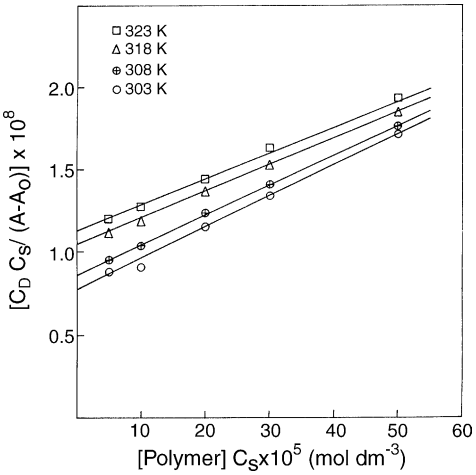

Fig. 4. Plot of $C_D C_S/A - A_0$ vs. C_S for pinacyanol chloride dye and *Klebsiella* K16 polymer interaction at different temperatures. Concentration of dye: 1.0×10^{-5} mol dm^{-3}. The temperatures: 303 K (◯), 308 K (⊕), 318 K (Δ), and 323 K (□).

measuring absorbance of the solution at the α band and also at the μ band. Absorbance at 600 nm (α band) increased with the increase in concentration of the added solvent–cosolvent and finally reached to the constant value corresponding to pure dye solution. Absorbance at 490 nm (μ band), however, decreased simultaneously and reached a constant minimum value. The results are shown in Figs. 5 and 6. At a certain concentration of added solvent, disappearance of the μ band indicated complete reversal of metachromasy. Amongst the alcohols, the ability of breaking the CPS–dye complex followed the order: methanol < ethanol < *n*-propanol. DMSO, DMF and urea were also found to be quite effective in causing reversal of metachromasy.

Fluorescence studies on interaction of the CPS with acridine orange dye were carried out with fixed concentration of dye and different concentration of CPS in aqueous media. The emission maximum of pure AO dye (λ_{em}) is 525.2 nm and the fluoresence intensity of dye was quenched gradually with increase in concentration of polymer, i.e., with enhanced P/D ratio which is shown in Fig. 7(a). By measuring the decrease in relative quantum yield of AO (excited at λ_{ex} 480 nm) at varying concentrations of polymer, the Stern–Volmer quenching constant, K_{SV}, has been calculated using the Stern–Volmer equation [18]:

$$\frac{\Phi_f^0}{\Phi_f} = 1 + K_{SV}[Q] \quad (2)$$

where Φ_f^0 and Φ_f are the relative quantum yields of fluorescence of AO alone and in the presence of polymer as quencher, respectively, and [Q] is the molar concentration of quencher polymer. The plots of Φ_f^0/Φ_f versus [Q] are linear with unit intercept and quenching or binding constant of dye and polymer, K_{SV} has been calculated from slope (Fig. 7(b)).

The quenching phenomenon was utilized for carrying out fluorometric titration where the fluorescence intensity at

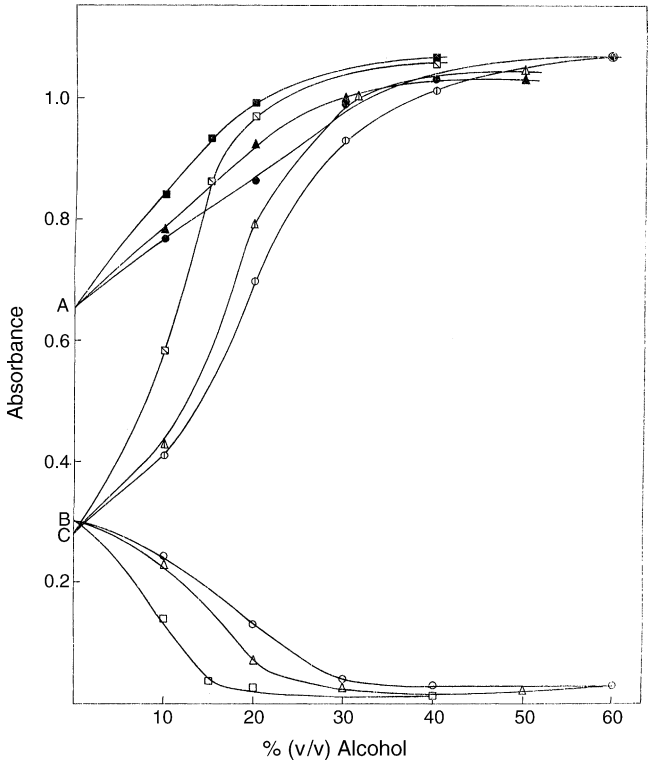

Fig. 5. Reversal of metachromasy in *Klebsiella* K16 CPS–pinacyanol chloride complex ([dye] = 1.0×10^{-5} mol dm^{-3}; P/D = 30) by the addition of methanol (◯), ethanol (Δ), and *n*-propanol (□) at 298 K. A: absorbance of pure dye at 600 nm; B: absorbance of dye–CPS complex at 490 nm; C: absorbance of dye–CPS complex at 600 nm.

525.2 nm was plotted against the volume of K16 polymer is shown in Fig. 3. Like spectrophotometic method, the equivalent weight of polymer was 670.88 and stoichiometry of the dye and polymer in its complex was found to be 1:1.02 from the point of intersection of two lines of fluorometric titration curves.

The equivalent weight obtained from the spectroscopic titration methods and conductance method were very close to each other and also found to be in good agreements with the calculated value from the structure of polymer, *Klebsiella* K16. The stoichiometric results obtained from the titration methods showed that dye and polymer interact with each other at more or less 1:1 ratio. The MacIntosh method also exhibited the same result.

At higher P/D values, a strong μ band appeared with repression of α and β bands and a blue shift of 110 nm was observed indicating strong metachromasy. The shape of metachromatic spectra depends on the conformation of the polyions as well as dye ion in solution. It has been reported [19,20] that polyglutamic acid produces a sharp single banded metachromatic spectrum in PCYN dye at acidic pH due to α-helical form of the polypeptide but it produces a broad multiple band spectrum in the dye as a result of random coil conformation at neutral pH. Pal and co-workers [21,22] also reported on such conformation influence on the spectrum of pinacyanol chloride dye by synthetic polyanions. In the case

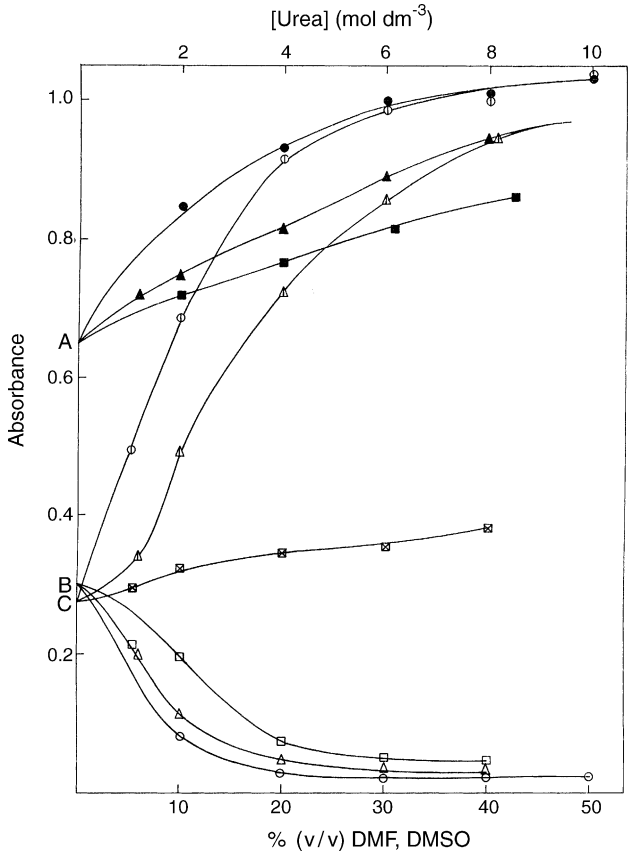

Fig. 6. Reversal of metachromasy in *Klebsiella* K16 CPS–pinacyanol chloride complex ([dye] = 1×10^{-5} mol dm^{-3}; P/D = 30) by the addition of DMF (◯), DMSO (Δ), and urea (□) at 298 K. A: absorbance of pure dye at 600 nm; B: absorbance of dye–CPS complex at 490 nm; C: absorbance of dye–CPS complex at 600 nm.

of *Klebsiella* K16 polymer, appearance of multiple banded broad spectra indicated that the polymer might have random coil structure in the solution and there was no significant change in conformation of the polymer since the shape of the spectra of the polymer remained unchanged at higher P/D value.

Metachromasy can be destroyed by different means and the process is termed as reversal of metachromasy. The concept of reversal of metachromasy is used to determine the stability of the metachromatic compound. Spectral studies of the metachromatic solutions in mixture of alcohol and water led to develop a new method to quantative estimate the relative stability of them. The extent of destruction of metachromatic compounds by alcohols was followed by the increasing absorbances of dye–polymer solution at the α band and decreasing absorbance at μ band simultaneously as a function of concentrations of alcohols and urea. Destruction of metachromasy was perhaps mainly due to breaking of hydrophobic bonds by alcohols and urea [6,23].

Mitra and Chakraborty [12] pointed out that two fluorescent dyes, AO and phenosafranin preferred to form a staggered aggregation on binding to *Klebsiella* K14 polymer from the spectofluorometric study. Since *Klebsiella* K16 belongs to same family of *Klebsiella* K14, so fluorescence quenching of the dye, AO by *Klebsiella* K16 at different P/D values, reflects a staggered aggregation of dye on binding to PK16.

The thermodynamic parameters were evaluated by considering simple equilibrium for the dye and polymer interaction. The decrease of K_c and ΔH with increase in temperature indicated that dye and polymer are associated due to electrostatic attraction which might be important initial factor in securing close association between the dye cations and the

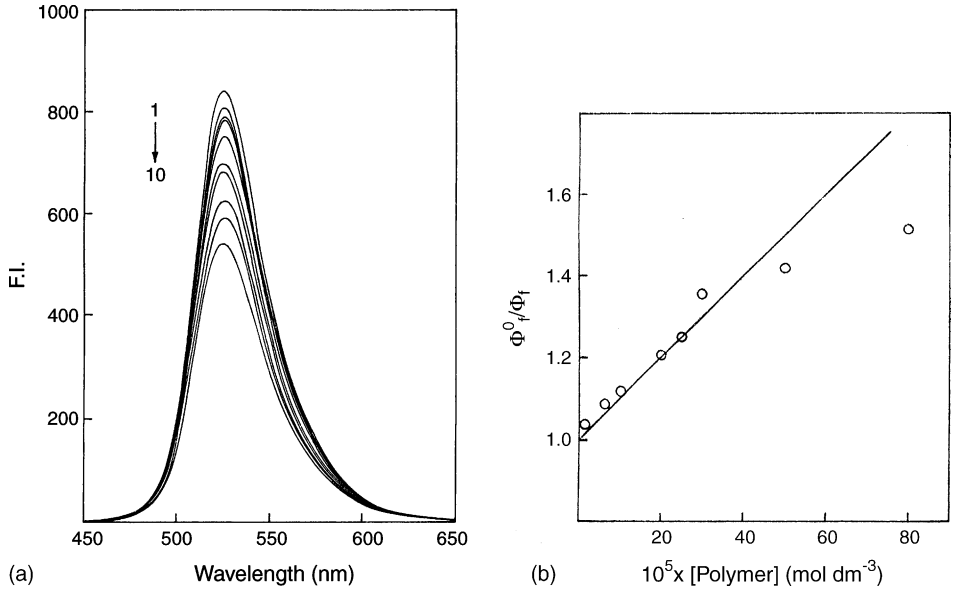

Fig. 7. (a) Emission spectra of acridine orange in presence of *Klebsiella* K16 polymer with different (P/D): (1) 0.0, (2) 1.0, (3) 2.0, (4) 6.0, (5) 10.0, (6) 20.0, (7) 25.0, (8) 30.0, (9) 50.0, and (10) 80.0 at 298 K. Concentration of dye = 1.0×10^{-5} mol dm^{-3}; λ_{ex} = 480 nm. (b) Stern–Volmer plot for the fluorescence quenching of acridine orange by *Klebsiella* K16 CPS. The concentration of dye = 1.0×10^{-5} mol dm^{-3}.

anionic site of the polyanions. The small negative value of ΔG falls within the range of reversible biological process suggests a non-chemical type of interaction. The negative value of ΔS, also indicated more ordered state of the ions due to aggregation. All these thermodynamic parameters suggested that there was interaction between the anionic sites of the polyanions and the dye counterions, resulting in induction of metachromasy. The electrostatic interaction between the cationic dye and the polyanion is exhibited by the loss of energetic symmetry in the chromophoric system which is the most important factor in the spectral shift.

Aggregation of planar dye like PCYN and AO on anionic polymer is expected to form card pack stacking, confirmed by blue shifted metachromasy transition. Stoichiometry results of 1:1 indicated that the glucouronic acid present in each repeating unit of the PK16 was the potential anionic sites in the polymer for interaction with the dye cations.

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